Maize H3C4 promoter combined with the first intron of rice actin, chimeric gene comprising it and transformed plant

The present invention relates to a new 5′ regulatory sequence allowing the expression, in monocotyledonous plants, of a sequence heterologous to the said regulatory sequence, encoding a protein of interest. The present invention also relates to a chimeric gene comprising the said regulatory sequence, a heterologous sequence encoding a protein of interest and a 3′ regulatory sequence allowing the expression of the protein of interest in a plant cell from a monocotyledonous plant, as well as a transformed monocotyledonous plant comprising the said chimeric gene and the means necessary for the transformation of plant cells and of plants.

Various promoters allowing the expression of sequences encoding proteins of interest in plants are known, are described in the literature, and have already allowed the development, to a commercial stage, of plants modified by genetic engineering. They are promoter sequences of genes which are expressed naturally in plants, in particular promoters of bacterial, viral or plant origin such as, for example, that of a gene for the ribulose bisphosphate carboxylase/oxygenase small subunit (US 4,962,028) or of a gene of a plant virus such as, for example, that

of cauliflower mosaic (US 5,352,605). Promoters allowing the expression of heterologous genes in plants are in particular described in the following patents and patent applications: US 5,086,169, EP 0 353 908, US 5,139,954, US 5,378,619, US 5,563,328, US 5,589,583, US 5,633,363, US 5,633,439, US 5,633,440, US 5,633,447, US 5,635,618, US 5,639,948 and US 5,639,952.

However, some of these promoters, and more particularly the promoters of plant origin, are not functional in monocotyledonous plants.

Arabidopsis sp. histone promoters described in patent application EP 0,507,698 are for example known which are particularly efficient for allowing the expression of a heterologous gene in dicotyledonous plants such as tobacco, oil seed rape or soya bean, which are not functional in monocotyledonous plants such as maize.

The rice actin promoter is a promoter known to allow the expression of heterologous genes in monocotyledonous plants (US 5,641,876). However, the problem of identifying new functional 5' regulatory sequences for the expression of heterologous sequences in monocotyledonous plants still remains.

The present invention relates to a new DNA sequence, a 5' regulatory element allowing the expression of a heterologous gene in a plant cell from a monocotyledonous plant, the said DNA sequence comprising, in the direction of transcription, a first

DNA sequence, which is a functional fragment of the sequence of the maize H3C4 promoter, and a second DNA sequence, which is a functional fragment of the sequence of the first intron of rice actin.

The sequence of the maize H3C4 promoter is in particular described by Brignon *et al.* (Plant. Mol. Biol., 22: 1007-1015, 1993). It is the *AluI* fragment of the maize H3C4 promoter, of about 1 kb, corresponding to bases -7 to -1029 relative to the ATG of the sequence encoding the maize histone H3C4.

The sequence of the first intron of rice actin is in particular described in patent US 5,641,876.

Functional fragment is understood according to the invention to mean any DNA sequence derived from the sequence of the maize H3C4 promoter or from the sequence of the first intron of rice actin, which reproduces the function of the sequence from which it is derived.

According to one embodiment of the invention, the functional fragment of the sequence of the maize H3C4 promoter comprises the DNA sequence described by the sequence identifier No. 1 (SEQ ID NO: 1) or a sequence homologous to the said sequence. Preferably, the functional fragment of the sequence of the maize H3C4 promoter consists of the DNA sequence described by the sequence identifier No. 1.

According to one embodiment of the invention,

the functional fragment of the first intron of rice actin comprises the DNA sequence described by the sequence identifier No. 2 (SEQ ID NO: 2) or a sequence homologous to the said sequence. Preferably, the functional fragment of the first intron of rice actin consists of the DNA sequence described by the sequence identifier No. 2.

The DNA sequence, a 5' regulatory element, according to the invention may comprise, in addition, between the first and second DNA sequences, neutral DNA fragments which are generally necessary for the construction of the sequence according to the invention. These are DNA fragments comprising up to 30 base pairs, preferably up to 20 base pairs. Neutral DNA fragments are understood according to the invention to mean DNA fragments which do not substantially modify the respective functions of the first and second DNA sequences of the sequence according to the invention.

According to a preferred embodiment of the invention, the DNA sequence according to the invention comprises the DNA sequence represented by the sequence identifier No. 3 (SEQ ID NO: 3) or a sequence homologous to the said sequence. More preferably, the sequence according to the invention consists of the DNA sequence represented by the sequence identifier No. 3.

"Homologue" is understood according to the invention to mean a DNA sequence representing one or more sequence modifications relative to the reference

DNA sequence described by the sequence identifier No. 1, 2 or 3, and reproducing the function of the abovementioned sequences. These modifications may be obtained according to the customary mutation techniques, or alternatively by choosing the synthetic oligonucleotides which may be used in the preparation of the said sequence by hybridization. Advantageously, the degree of homology will be at least 70% relative to the reference sequence, preferably at least 80%, more preferably at least 90%.

The present invention also relates to a chimeric gene (or an expression cassette) comprising a coding sequence as well as heterologous regulatory elements at the 5′ and 3′ positions capable of functioning in plant cells from monocotyledonous plants, in which the 5′ regulatory elements comprise the DNA sequence according to the invention defined above.

"Plant cell" is understood to mean according to the invention any cell derived from a monocotyledonous plant and capable of constituting undifferentiated tissues such as calli, differentiated tissues such as embryos, monocotyledonous plant portions, monocotyledonous plants or seeds.

"Monocotyledonous plant" is understood according to the invention to mean any differentiated multicellular organism capable of photosynthesis, more particularly crop plants intended or otherwise as

animal feed or for human consumption, such as for example wheat, barley, oats, rice, maize, sorghum, sugar cane and the like.

According to the invention, it is also possible to use, in combination with the regulatory promoter sequence according to the invention, other regulatory sequences, which are situated between the promoter and the coding sequence, such as the sequences encoding transit peptides, either single, or double, and in this case optionally separated by an intermediate sequence, that is to say comprising, in the direction of transcription, a sequence encoding a transit peptide for a plant gene encoding a plastid localization enzyme, a portion of sequence of the mature N-terminal portion of a plant gene encoding a plastid localization enzyme, and then a sequence encoding a second transit peptide for a plant gene encoding a plastid localization enzyme consisting of a portion of sequence of the mature N-terminal portion of a plant gene encoding a plastid localization enzyme, as described in application EP 0,508,909. As transit peptide, there may also be mentioned the signal peptide for the tobacco PR-1a gene described by Cornelissen *et al*.

As regulatory terminator or polyadenylation sequence, there may be used any corresponding sequence of bacterial origin, such as for example the *Agrobacterium tumefaciens* nos terminator, or

alternatively of plant origin, such as for example a histone terminator as described in application EP 0,633,317.

The coding sequence of the chimeric gene according to the invention may comprise any sequence encoding the protein of interest which it is desired to express in a plant cell or a monocotyledonous plant.

This may be a gene encoding a selectable marker such as a gene conferring on the transformed monocotyledonous plant new agronomic properties, or a gene for enhancing the agronomic quality of the transformed monocotyledonous plant.

Among the genes encoding selectable markers, there may be mentioned genes for resistance to antibiotics, genes for tolerance to herbicides (bialaphos, glyphosate or isoxazoles), genes encoding easily identifiable enzymes such as the enzyme GUS, genes encoding pigments or enzymes regulating the production of pigments in the transformed cells. Such selectable marker genes are in particular described in patent applications WO 91/02071 and WO 95/06128.

Among the genes conferring new agronomic properties on transformed monocotyledonous plants, there may be mentioned the genes conferring tolerance to certain herbicides, those conferring tolerance to certain insects, those conferring tolerance to certain diseases and the like. Such genes are in particular described in patent applications WO 91/02071 and

WO 95/06128.

As regulatory terminator or polyadenylation sequence, there may be used any corresponding sequence of bacterial origin, such as for example the *Agrobacterium tumefaciens* nos terminator, or alternatively of plant origin, such as for example a histone terminator as described in application EP 0,633317.

The present invention is particularly appropriate for the expression of genes conferring tolerance to certain herbicides on transformed plant cells and on transformed monocotyledonous plants. Among the genes conferring tolerance to certain herbicides, there may be mentioned the *Bar* gene conferring tolerance to bialaphos, the gene encoding an appropriate EPSPS conferring resistance to herbicides having EPSPS as target, such as glyphosate and its salts (US 4,535,060, US 4,769,061, US 5,094,945, US 4,940,835, US 5,188,642, US 4,971,908, US 5,145,783, US 5,310,667, US 5,312,910, US 5,627,061, US 5,633,435, FR 2,736,926), the gene encoding glyphosate oxydoreductase (US 5,463,175), or alternatively a gene encoding an HPPD conferring tolerance to herbicides having HPPD as target, such as the isoxazoles, in particular isoxafutole (FR 95 06800, FR 95 13570), the diketonitriles (EP 496 630, EP 496 631) or the triketones, in particular sulcotrione (EP 625 505, EP 625 508, US 5,506,195). Such genes encoding an HPPD

conferring tolerance to herbicides having HPPD as target are described in patent application WO 96/38567 and in unpublished patent application FR 97 14264, filed on 7 November 1997, whose content is incorporated herein by reference.

Among the genes encoding an appropriate EPSPS conferring resistance to herbicides having EPSPS as target, there may be mentioned more particularly the gene encoding a plant EPSPS, in particular from maize, having two mutations 102 and 106, which is described in patent application FR 2,736,926, called hereinafter double-mutant EPSPS, or alternatively the gene encoding an EPSPS isolated from *Agrobacterium* which is described by the sequences ID 2 and ID 3 of patent US 5,633,435, called hereinafter CP4.

Among the genes encoding an HPPD conferring tolerance to herbicides having HPPD as target, there may be mentioned more particularly the HPPD from *Pseudomonas* and that from *Arabidopsis*, which are described in patent application WO 96/38567.

In the case of the genes encoding EPSPS or HPPD, and more particularly for the above genes, the sequence encoding these enzymes is advantageously preceded by a sequence encoding a transit peptide, in particular the transit peptide called optimized transit peptide described in patents US 5,510,471 or US 5,633,448 whose content is incorporated herein by reference.

According to a preferred embodiment of the invention, the chimeric gene according to the invention comprises, in the direction of transcription, a 5′ regulatory sequence according to the invention as defined above, functionally linked to a sequence encoding a fusion protein transit peptide/protein of interest, functionally linked to a 3′ regulatory sequence, the different elements of the chimeric gene being defined above, the protein of interest being preferably an enzyme conferring tolerance to certain herbicides, more preferably enzymes of the EPSPS or HPPD type defined above.

Sequences encoding fusion proteins transit peptide/EPSPS, and more particularly OTP/double-mutant EPSPS are in particular described in patents US 4,940,835, US 5,633,448 and FR 2 736 926.

For the fusion protein OTP/CP4, persons skilled in the art will know how to construct the corresponding gene by taking the sequence encoding the CP4 described in patent US 5,633,435 and by following the procedure described in patents US 4,940,835, US 5,633,448 and FR 2,736,926 or in the examples below. The present invention also relates to a chimeric gene comprising, in the direction of transcription, an appropriate 5′ regulatory sequence to ensure the expression of a heterologous gene in a plant cell, functionally linked to a sequence encoding a fusion protein OTP/CP4, functionally linked to a 3′ regulatory

sequence. The 5' regulatory elements comprise not only the 5' regulatory elements according to the invention defined above, but also all the appropriate regulatory elements for allowing the expression of heterologous genes in plant cells from monocotyledonous or dicotyledonous plants which are known to a person skilled in the art or of the future, and in particular those described above.

The sequences encoding fusion proteins transit peptide/HPPD are described in patent application WO 96/38567.

The present invention also relates to a cloning or expression vector for the transformation of a plant cell or of a monocotyledonous plant, the transformed plant cells and plants containing at least one chimeric gene as defined above. The vector according to the invention comprises, in addition to the above chimeric gene, at least one replication origin. This vector may consist of a plasmid, a cosmid, a bacteriophage or a virus, which are transformed by introducing the chimeric gene according to the invention. Such vectors for transforming plant cells and monocotyledonous plants are well known to a person skilled in the art and are widely described in the literature. Preferably, the vector for transforming plant cells or plants according to the invention is a plasmid.

The subject of the invention is also a method

of transforming plant cells by integrating at least one nucleic acid fragment or a chimeric gene as defined above, which transformation may be obtained by any appropriate known means with the vector according to the invention.

A series of methods consists in bombarding cells or cellular tissues with particles to which DNA sequences are attached. Another series of methods consists in using, as means of transfer into the plant, a chimeric gene inserted into an *Agrobacterium tumefaciens* Ti plasmid or an *Agrobacterium rhizogenes* Ri plasmid. Other methods may be used, such as microinjection or electroporation, or alternatively direct precipitation by means of PEG.

Persons skilled in the art will choose the appropriate method according to the nature of the plant cell or of the plant.

The subject of the present invention is also the plant cells or plants transformed and which contain at least one chimeric gene according to the invention defined above.

The subject of the present invention is also the plants containing transformed cells, in particular the plants regenerated from transformed cells. The regeneration is obtained by any appropriate method which depends on the nature of the species.

For the methods of transforming plant cells and of regenerating monocotyledonous plants, there may

be mentioned in particular Gordon-Kamm, W.J. *et al.* (*Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants,* The Plant Cell, vol. 2, 603-618, July 1990), whose content is incorporated herein by reference, and the following patents and patent applications: US 5,177,010, US 5,187,073, EP 267,159, EP 604 662, EP 672 752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US 5,204,253, US 5,405,765, EP 442 174, EP 486 233, EP 486 234, EP 539 563, EP 674 725, WO 91/02071 and WO 95/06128.

The present invention also relates to the transformed plants derived from the culture and/or the crossing of the above regenerated plants, as well as the seeds of transformed plants.

In the case where the chimeric gene according to the invention comprises a sequence encoding an enzyme conferring tolerance to a particular herbicide, the present invention also relates to a method of controlling weed in an area of a field comprising seeds or plants transformed with the said chimeric gene according to the invention, which method consists in applying to the said area of the field a dose of the said particular herbicide which is toxic to the said weed, without, however, substantially affecting the seeds or plants transformed with the said chimeric gene according to the invention comprising the said sequence

encoding an enzyme conferring tolerance to the said particular herbicide.

The present invention also relates to a method of culturing the plants transformed according to the invention with a chimeric gene according to the invention comprising a sequence encoding an enzyme conferring tolerance to a particular herbicide defined above, which method consists in planting the seeds of the said transformed plants in an area of a field which is appropriate for the culture of the said plants, in applying to the said area of the said field a dose of the said particular herbicide which is toxic to weeds should weeds be present, without substantially affecting the said seeds or the said transformed plants, and then in harvesting the cultivated plants when they reach the desired maturity and optionally in separating the seeds from the harvested plants.

In the above two methods, the application of the particular herbicide may be made according to the invention before sowing, before emergence and after emergence of the crop.

Advantageously, the enzyme for tolerance to a herbicide is an appropriate EPSPS, and in this case the herbicide is glyphosate or its salts, or the enzyme is an HPPD and the herbicide is chosen from the isoxazoles, in particular isoxafutole, the diketonitriles or the triketones, in particular sulcotrione.

The examples below make it possible to illustrate the invention without seeking to limit its scope.

1. Construction of a chimeric gene with a sequence encoding an HPPD:

The plasmids below are prepared so as to create an expression cassette comprising a maize H3C4 histone promoter combined with the untranslated 5′ region of the first intron of the rice actin gene (*ActI*) described by Mc Elroy D. *et al.* (Plant Molecular Biology 15: 257-268 (1990)) directing the expression of the gene OTP-HPPD of *Pseudomonas fluorescens*.

pRPA-RD-195

The plasmid pRPA-RD-195 is a derivative of the plasmid pUC-19 which contains a modified multiple cloning site. The complementary oligonucleotides 1 and 2 below are hybridized at 65°C for 5 minutes, followed by a slow cooling down to 30°C over 30 minutes:

Oligo 4: 5' AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC CTCTAGAGTC GACCTGCAGG CATGC 3'

Oligo 5: 5' CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC TAGAGG 3'

The hybridized oligonucleotides are made double-stranded using the Klenow fragment of DNA polymerase I of *E. coli* to extend the 3′ ends of each oligo using the standard conditions recommended by the manufacturer (New England Biolabs). The double-stranded oligo obtained is then linked in the plasmid pUC-19

previously digested with the restriction enzymes *EcoRI* and *HindIII* and made blunt-ended using the Klenow fragment of DNA polymerase I of *E. coli*. A cloning vector is thus obtained which comprises a multiple cloning site so as to facilitate the introduction of expression cassettes into a plasmid vector of *Agrobacterium tumefaciens* (Figure 1).

pRPA-RD-2010:

Insertion of the sequence "H4A748 promoter-OTP-double mutant EPSPS gene" of pRPA-RD-159 into the plasmid pRPA-RD-195.

The plasmid pRPA-RD-195 is digested with the restriction enzyme *SacI* and dephosphorylated with calf intestinal alkaline phosphatase (New England Biolabs). The plasmid pRPA-RD-173 (described in patent FR 2,736,926) is digested with the restriction enzyme *SacI* and the DNA fragment containing the EPSPS gene is purified and linked into the plasmid pRPA-RD-195 prepared above. The clone obtained contains several unique restriction sites flanking the double-mutant EPSPS gene.

pRPA-RD-1002

Creation of an expression cassette OTP-HPPD for use in monocotyledonous plants. The plasmid pRP-P contains the optimized transit peptide (OTP) linked to the HPPD of *Pseudomonas fluorescens* followed by the polyadenylation site of nopaline synthase as described in patent application WO 96/38567. The components of

the plasmid pRP-P are the following:

- the optimized transit peptide (OTP) described in patents US 5,510,471 and US 5,633,448; this OTP consisting of 171 bp of the *Helianthus annuus* ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit transit peptide (Waksman G. *et al*. 1987. Nucleics Acids Res. 15: 7181) which are followed by the 66 bp of the mature portion of the *Zea mays* ribulose 1,5-bisposphate carboxylase/oxygenase small subunit (Lebrun *et al*. 1987. Nucleics Acids Res. 15: 4360) which are themselves followed by the 150 bp of the *Zea mays* ribulose 1,5-bisphosphate carboxylase/oxygenase small subunit transit peptide (Lebrun *et al*. 1987. Nucleics Acids Res. 15: 4360); the combination is therefore 387 bp;

- the coding region of the HPPD of *Pseudomonas fluorescens* described in patent application WO 96/38567; and

- the nopaline synthase (nos) terminator gene (polyadenylation zone of the *nos* gene isolated from pTi 37, 250 bp; Bevan M. *et al*. Nucleics Acids Res. 11: 369-385).

The plasmid pRP-P is digested with the restriction enzyme *BstEII*, treated with the Klenow fragment of DNA polymerase I of *E. coli* in order to make the fragment blunt-ended, and followed by digestion with the restriction enzyme *NcoI*. The DNA fragment obtained, containing the coding region OTP-

HPPD about 1.5 kb, is then purified. The plasmid pRPA-RD-2010 obtained above is digested with the restriction enzyme *BlpI*, treated with the Klenow fragment of DNA polymerase I of *E. coli* in order to obtain a blunt-ended fragment, and then digested with the restriction enzyme *NcoI*. The DNA fragment obtained, comprising the sequences of the plasmid vector, the H3C4 promoter combined with the untranslated 5′ region and the first intron of the rice actin gene, is purified and the NOS polyadenylation site is purified. The two DNA fragments purified are linked so as to create an expression cassette OTP-HPPD comprising the maize H3C4 histone promoter (Brignon *et al.*) combined with the 5′ untranslated region and the first intron of the rice actin gene (*ActI*) (Act1 5′ UTR + intron 1) in order to control the expression of the coding region OTP-HPPD incorporating the NOS polyadenylation site (NOS polyA) (Figure 2).

2. Construction of a chimeric gene with a sequence encoding the double-mutant EPSPS:

pRPA-RD-1010:

Creation of an expression cassette OTP-double mutant EPSPS for use in monocotyledonous plants.

The plasmid pRPA-RD-109 contains the β-glucuronidase (GUS) gene of *E. coli* controlled by the maize H3C4 histone promoter (Brignon *et al.*) combined with the 5′ untranslated region and the first intron of the rice actin gene (*ActI*) described by Mc Elroy D.

et al. (Plant Molecular Biology 15: 257-268, 1990). A diagram of this plasmid is represented in Figure 3. The plasmid pRPA-RD-109 is digested with the restriction enzymes *NcoI* and *EcoRI*, and the large DNA fragment (about 5 kb) containing the vector sequence, the GUS gene and the NOS polyadenylation site is purified. The plasmid pRPA-RD-2010 is digested with the restriction enzymes *NcoI* and *EcoRI*, and the DNA fragment (about 1.6 kb) containing the H3C4 promoter combined with the 5′ untranslated region and the first intron of the rice actin gene (*ActI*) is purified. The two DNA fragments purified are linked in order to create an expression cassette OTP-double mutant EPSPS comprising the maize H3C4 histone promoter (Brignon *et al.*) combined with the 5′ untranslated region and the first intron of the rice actin gene (*ActI*) in order to control the expression of the coding region OTP-double mutant EPSPS incorporating the NOS polyadenylation site.

3. Construction of a chimeric gene for tolerance to phosphinothricin (*bar* gene):

The phosphinothricin acetyl transferase (PAT) encoded by the *bar* gene is an enzyme which inactivates a herbicide, phosphinothricin (PPT). PPT inhibits the synthesis of glutamine and causes a rapid accumulation of ammonia in the cells, leading to their death (Tachibana *et al.* 1986).

The plasmid used to introduce the tolerance to phosphinothricin as selection agent is obtained by

inserting the chimeric gene pDM 302 into the vector pSP72 of 2462 bp, marketed by Promega Corp. (Genbank/DDBJ database accession number X65332) and containing the gene for resistance to ampicillin.

The plasmid pDM 302 of 4700 bp has been described by Cao, J., *et al.* Plant Cell Report 11: 586-591 (1992).

The various components of this plasmid are:

- the promoter of the rice actin gene described by Mc Elroy D. *et al.* Plant Molecular Biology 15: 257-268 (1990) consisting of 840 bp;
- the first exon of the rice actin gene consisting of 80 bp;
- the first intron of the rice actin gene consisting of 450 bp;
- the region encoding the *bar* gene of 600 bp excised from the plasmid pIJ41404 described by White J. *et al.* Nuc. Acids Res. 18: 1862 (1990);
- the terminator of the nopaline synthase (nos) gene (polyadenylation zone of the *nos* gene isolated from pTi 37, 250 bp; (Bevan M. *et al.* Nucleics Acids Res. 11: 369-385).

4. Transformation of maize cells:

The particle bombardment technique is used to introduce the genetic construct. The plasmids are purified on a Qiagen column and coprecipitated on M10 tungsten particles according to the Klein method (Nature 327: 70-73, 1987).

A mixture of metal particles, of the plasmid pRPA-RD-1002 and of the plasmid of Example 3 which are described above, is then bombarded onto embryogenic maize cells according to the protocol described by Gordon-Kamm, W.J. *et al.* (*Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants*, The Plant Cell, vol. 2, 603-618, July 1990).

5. Regeneration and use of the *bar* gene as selection agent:

The bombarded calli are selected on glufosinate until green sectors appear. The glufosinate-resistant positive calli are then converted to somatic embryos, and then placed under conditions which promote germination according to the operating conditions described by Gordon-Kamm, W.J. *et al.* (*Transformation of Maize Cells and Regeneration of Fertile Transgenic Plants*, The Plant Cell, vol. 2, 603-618, July 1990). The young plants are transferred to a greenhouse for the production of seeds.

6. Analysis of the progeny of the transformed plants:

The transformed plants obtained above are assumed in part to be transgenic, comprising a heterologous gene encoding OTP/HPPD conferring tolerance to isoxazoles such as isoxafutole. These transformed plants produced pollen, which fertilized ovules from a nontransgenic wild-type maize. The seeds obtained are selected on sand after treating with isoxaflutole.

The selection protocol is the following:

800 ml of Fontainebleau sand are placed in a tub of sides 15 x 20 cm. These tubs are then sprinkled with water and kept moist by supplying a nutrient solution consisting of 5 ml of Quinoligo (Quinoline) per litre of water. Twenty maize seeds are placed in the tubs, which are then treated with isoxaflutole by spraying at a rate of 100 g of active material per hectare (300 µg of active material per tub). The tubs are then cultured in a greenhouse. The phytotoxicity is determined 14 [lacuna] after planting. According to the above conditions, the nontransformed plants exhibit 100% phytotoxicity whereas the transformed plants exhibit no phytotoxicity.

A comparative study was carried out with 20 maize lines transformed according to the invention and 20 maize lines transformed with a corresponding gene for which the sequence encoding the first intron of rice actin has been replaced with the sequence encoding the maize adh I intron. After treating by spraying very high doses of isoxafutole at a rate of 200 g of active material per hectare (600 µg of active material per tub), the following results are obtained:

Rice actin intron according to the invention: 8/20 lines are tolerant

Maize adh I intron: 3/20 lines are tolerant

The results above demonstrate that the combination of the maize H3C4 promoter with the first

intron of rice actin according to the invention substantially enhances the expression of a protein of interest in transformed monocotyledonous plants compared with the combination of the same maize H3C4 promoter with another intron of the state of the art.

SEQUENCE LISTING

(iii) NUMBER OF SEQUENCES: 5

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1021 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: maize H3C4 promoter
(B) LOCATION: 1..1021

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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CTTATGTGCA CCATTTACTG TAATGCATAA TCATTTAATT GAATAGCAAA CTTTTCTATT     60
ACTTCTTTAC TAACATAATT CTTGGTTTTA AAATTCAGTC CTCAACATTC ATTGCTCAAG    120
TATAAGTTGA GACTGTCAAA ATTTACTATT TTATTTCTTC ATATTTTTTT TCCTTATACA    180
CATTTTGGGC CTTACAATCC ATCATCTATA TCCATCCTTT CCGGTGTCCT CTAAAAGATT    240
CCATCCTCTG AATCTTATTC CTCTCCAATA ACGTTCTCTA AATCAGGTCT CTATAAGCAA    300
TACCTATATT AGAGACATTT TTTATTTTTT GTACATACAT ATTTGTCATA CTCTCAAATG    360
CATTATACAT ATTTAGTTTT ACTAAACCGA TTATTTAAAG TATTCAAACG GATGAAGAAC    420
TGTTTAGATA AATTCTATAT ATAGAGAATC CAGTAGCGTT CTCTAAATTT AGATGATTAT    480
TTAGAGGACG CTGTTAGAAA ACGTAAAAAA TTCTTTGATT ATTTATATTT AGGGTAGAGT    540
AGCCTTTATG CTTTATAGAT CTTTGGTGGA CCCAGCCTTA TACCGGTTAT TTTCGCGATT    600
GCGCCTCTCA TTTTCACTCC AGCGCCCCAC ATTTTCACGT TTTCACCGAA GCGCCCAGCC    660
TGCCTAACCA ACAAATTGGT ACGGTGGCGC GGTTTTCAAA AGAAGTCGGA AACCATCTGC    720
ACCCACCGAC TAGTAGGCCC TCGGATCCTC CCTGATTAAG TCCTAGCCAA TAGGAGCCCA    780
GAACCACCCA TCACGCGGAT CGTCCCTACG CTTCCACCTC ATCGGCGCCG TCCATCTCCA    840
TCCAACACCT ATTCCGTTAC CTTGCCCATC CTCCGAAAAA ATTCTCGGCT CGCGCTCCGC    900
ACCTACTACA AATACCCATC CCATCACGAC GCATCGCATC ACTGCCAAAT CCCCCAGAAA    960
ATCAACACCT CCCAATTCCA CGCTGCCACC AACTCGCCGT CCTCCGCGCC AAGCACCAAA   1020
G                                                                   1021
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 454 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(ix) FEATURE:
(A) NAME/KEY: intron 1 of rice actin
(B) LOCATION: 1..454

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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GTAACCACCC CGCCCCTCTC CTCTTTCTTT CTCCGTTTTT TTTTTCGTCT CGGTCTCGAT      60
CTTTGGCCTT GGTAGTTTGG GTGGGCGAGA GCGGCTTCGT CGCCCAGATC GGTGCGCGGG     120
AGGGGCGGGA TCTCGCGGCT GGCGTCTCCG GGCGTGAGTC GGCCCGGATC CTCGCGGGGA     180
ATGGGGCTCT CGGATGTAGA TCTGATCCGC CGTTGTTGGG GGAGATGATG GGGCGTTTAA     240
AATTTCGCCA TGCTAAACAA GATCAGGAAG AGGGGAAAAG GGCACTATGG TTTATATTTT     300
TATATATTTC TGCTGCTGCT CGTCAGGCTT AGATGTGCTA GATCTTTCTT TCTTCTTTTT     360
GTGGGTAGAA TTTGAATCCC TCAGCATTGT TCATCGGTAG TTTTTCTTTT CATGATTTGT     420
GACAAATGCA GCCTCGTGCG GAGCTTTTTT GTAG                                 454
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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1565 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: maize H3C4 promoter
(B) LOCATION: 27..1047

(ix) FEATURE:
(A) NAME/KEY: intron 1 of rice actin
(B) LOCATION: 1102..1555

(ix) FEATURE:
(A) NAME/KEY: synthetic cloning site
(B) LOCATION: 1..26

(ix) FEATURE:
(A) NAME/KEY: synthetic binding sequence
(B) LOCATION: 1048..1069

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
GAATTCCTGC AGGTCGACGG ATCCCCCTTA TGTGCACCAT TTACTGTAAT GCATAATCAT     60
TTAATTGAAT AGCAAACTTT TCTATTACTT CTTTACTAAC ATAATTCTTG GTTTTAAAAT    120
TCAGTCCTCA ACATTCATTG CTCAAGTATA AGTTGAGACT GTCAAAATTT ACTATTTTAT    180
TTCTTCATAT TTTTTTTCCT TATACACATT TTGGGCCTTA CAATCCATCA TCTATATCCA    240
TCCTTTCCGG TGTCCTCTAA AAGATTCCAT CCTCTGAATC TTATTCCTCT CCAATAACGT    300
TCTCTAAATC AGGTCTCTAT AAGCAATACC TATATTAGAG ACATTTTTTA TTTTTTGTAC    360
ATACATATTT GTCATACTCT CAAATGCATT ATACATATTT AGTTTTACTA AACCGATTAT    420
TTAAAGTATT CAAACGGATG AAGAACTGTT TAGATAAATT CTATATATAG AGAATCCAGT    480
AGCGTTCTCT AAATTTAGAT GATTATTTAG AGGACGCTGT TAGAAAACGT AAAAAATTCT    540
TTGATTATTT ATATTTAGGG TAGAGTAGCC TTTATGCTTT ATAGATCTTT GGTGGACCCA    600
GCCTTATACC GGTTATTTTC GCGATTGCGC CTCTCATTTT CACTCCAGCG CCCCACATTT    660
TCACGTTTTC ACCGAAGCGC CCAGCCTGCC TAACCAACAA ATTGGTACGG TGGCGCGGTT    720
TTCAAAAGAA GTCGGAAACC ATCTGCACCC ACCGACTAGT AGGCCCTCGG ATCCTCCCTG    780
ATTAAGTCCT AGCCAATAGG AGCCCAGAAC CACCCATCAC GCGGATCGTC CCTACGCTTC    840
CACCTCATCG GCGCCGTCCA TCTCCATCCA ACACCTATTC CGTTACCTTG CCCATCCTCC    900
GAAAAAATTC TCGGCTCGCG CTCCGCACCT ACTACAAATA CCCATCCCAT CACGACGCAT    960
CGCATCACTG CCAAATCCCC CAGAAAATCA ACACCTCCCA ATTCCACGCT GCCACCAACT   1020
CGCCGTCCTC CGCGCCAAGC ACCAAAGGAA TTGGCCGCCA CCGCGGTGGA GCTCCTCCCC   1080
CCTCCCCCTC CGCCGCCGCC GGTAACCACC CCGCCCCTCT CCTCTTTCTT TCTCCGTTTT   1140
TTTTTTCGTC TCGGTCTCGA TCTTTGGCCT TGGTAGTTTG GGTGGGCGAG AGCGGCTTCG   1200
TCGCCCAGAT CGGTGCGCGG GAGGGGCGGG ATCTCGCGGC TGGCGTCTCC GGGCGTGAGT   1260
CGGCCCGGAT CCTCGCGGGG AATGGGGCTC TCGGATGTAG ATCTGATCCG CCGTTGTTGG   1320
GGGAGATGAT GGGGCGTTTA AAATTTCGCC ATGCTAAACA AGATCAGGAA GAGGGGAAAA   1380
GGGCACTATG GTTTATATTT TTATATATTT CTGCTGCTGC TCGTCAGGCT TAGATGTGCT   1440
AGATCTTTCT TTCTTCTTTT TGTGGGTAGA ATTTGAATCC CTCAGCATTG TTCATCGGTA   1500

GTTTTTCTTT TCATGATTTG TGACAAATGC AGCCTCGTGC GGAGCTTTTT TGTAGGTAGA   1560
CC ATG                                                              1565
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 85 base pairs
(B) TYPE: synthetic oligonucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
AGGGCCCCCT AGGGTTTAAA CGGCCAGTCA GGCCGAATTC GAGCTCGGTA CCCGGGGATC       60
CTCTAGAGTC GACCTGCAGG CATGC                                             85
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: synthetic oligonucleotide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
CCCTGAACCA GGCTCGAGGG CGCGCCTTAA TTAAAAGCTT GCATGCCTGC AGGTCGACTC       60
TAGAGG                                                                  66
```